SMOKE SIGNALS: CAN A CIGARETTE HABIT BE READ FROM A WOMAN'S MENSTRUAL BLOOD?

A Proposed Cross-Sectional Study of Oxidative Stress Biomarkers and Endometrial Cell Gene Expression in Self-Collected Menstrual Effluent

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ABSTRACT

Background: Cigarette smoking is a well-established insult to female reproductive health, inducing systemic oxidative stress, hormonal dysregulation, and epigenetic alterations in ovarian and endometrial tissues. Despite this, no published study has used menstrual blood as the primary diagnostic medium to assess smoking-related reproductive damage — a significant gap given that menstrual effluent contains shed endometrial cells, endometrial stem cells, and locally derived metabolites that may capture the uterine biochemical environment far more directly than peripheral serum.

Objective: This paper proposes and justifies a cross-sectional study to determine whether smoking status can be detected through oxidative stress markers and gene expression signatures in self-collected menstrual blood.

Methods: Sixty reproductive-age women (30 active smokers, 30 non-smokers) will be recruited. Menstrual blood will be self-collected via menstrual cups during days 1–2 of the menstrual cycle. Biochemical assays will quantify malondialdehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD). RNA extracted from shed endometrial cells will be analysed by RT-qPCR for expression of *CXCL12*, *FGF2*, *HOXA10*, *CYP1A1*, *NRF2*, and *HMOX1*. Cotinine concentration will serve as an objective smoking verification marker.

Anticipated Results: Smokers are expected to exhibit significantly elevated MDA and suppressed antioxidant enzyme activity in menstrual effluent, alongside dysregulated endometrial receptivity gene expression, compared to non-smokers.

Significance: If confirmed, menstrual blood would represent a novel, non-invasive, cyclic-access biofluid for monitoring reproductive toxicity from smoking — democratising gynaecological surveillance beyond the clinical setting.

KEYWORDS

Menstrual blood · Cigarette smoking · Oxidative stress · Endometrial gene expression · Reproductive biomarkers · Non-invasive diagnostics · Menstrual self-collection

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1. INTRODUCTION

Every month, the human body performs what amounts to a controlled demolition and rebuilding of the endometrium — a process that expels shed epithelial cells, stromal fragments, endometrial stem cells, inflammatory cytokines, and a rich biochemical milieu into the menstrual effluent. For most of recorded medical history, this fluid has been regarded as biological waste and discarded accordingly. Recent research, however, is beginning to reframe menstrual blood as a diagnostically underutilised biofluid — one capable of yielding cellular and molecular information about the uterine environment without the need for biopsy, sedation, or clinical intervention (Patel et al., 2018; Allaire et al., 2023).

In parallel, the reproductive consequences of cigarette smoking are now extensively documented. Women who smoke face elevated risks of infertility, implantation failure, miscarriage, and premature ovarian insufficiency (Practice Committee of the American Society for Reproductive Medicine, 2012; Broekmans et al., 2009). The biochemical mechanisms underlying this damage centre on oxidative stress: the thousands of reactive compounds in cigarette smoke exhaust cellular antioxidant defences, elevate reactive oxygen species (ROS), and impose both structural (lipid peroxidation, DNA strand breaks) and regulatory (gene silencing via aberrant methylation, transcriptional dysregulation) damage across multiple reproductive tissues (Agarwal et al., 2012; Soares et al., 2007).

Yet despite this convergence of two rich research areas — menstrual blood diagnostics and smoking-related reproductive toxicology — no published study, to our knowledge, has used menstrual blood as the primary sample matrix to interrogate the biochemical or transcriptomic fingerprint of cigarette smoking in the female reproductive tract. Studies of smoking's endometrial effects have predominantly relied on serum measurements, endometrial biopsy, or

follicular fluid sampled invasively during assisted reproductive technology (ART) cycles (Freour et al., 2008; Howe et al., 2022). Each of these methods either fails to capture the uterine microenvironment specifically (serum), requires clinical infrastructure (biopsy), or restricts the study population to women already undergoing fertility treatment (follicular fluid), introducing profound sampling biases.

This paper argues that menstrual blood is an overlooked but exceptionally well-positioned sample source for this research question. Menstrual effluent provides direct access to shed endometrial cells at their source; it is obtainable monthly, non-invasively, in any setting; and the endometrial stem cells it contains retain molecular signatures of the uterine tissue from which they originate (Meng et al., 2007; Ulrich et al., 2014). If oxidative stress markers in menstrual blood differ significantly between smokers and non-smokers, and if endometrial cell gene expression in this fluid reflects smoking-induced transcriptional dysregulation, then menstrual blood would represent not only a novel research finding but a potentially transformative clinical tool.

The primary hypothesis of this study is: Active cigarette smokers will exhibit significantly elevated levels of malondialdehyde (MDA), and significantly reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) activity in menstrual blood, relative to non-smoking controls, alongside differential expression of endometrial receptivity and antioxidant response genes in shed endometrial cells.

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2. LITERATURE REVIEW AND BACKGROUND

2.1 Menstrual Blood as a Diagnostic Biofluid

The scientific rehabilitation of menstrual blood as a diagnostic resource has accelerated significantly over the past decade. Meng et al. (2007) were among the first to characterise menstrual blood-derived endometrial stem cells (MenSCs), establishing that the menstrual effluent contains a reproducible population of clonogenic, multipotent stromal cells with properties analogous to bone marrow mesenchymal stem cells. Unlike peripheral blood or urine, these cells carry tissue-specific transcriptional and epigenetic signatures of the endometrial environment (Ulrich et al., 2014).

Research into the diagnostic potential of menstrual blood has since expanded across multiple pathologies. In endometriosis, MenSCs from affected individuals demonstrate distinct proliferative behaviours and gene expression profiles compared to controls, raising the possibility of endometriosis diagnosis without laparoscopy (Nnoaham et al., 2011). Researchers have developed prototype lateral flow devices that detect HMGB1 — a protein elevated in the menstrual blood of endometriosis patients — akin to a home pregnancy test (D'Hooghe et al.,

2009). High-risk HPV has also been detected with high sensitivity and specificity in self-collected menstrual blood, suggesting utility in cervical cancer screening (Crosbie et al., 2019). Studies on uterine fibroids, polycystic ovary syndrome (PCOS), and hormonal imbalances have similarly found measurable differences in menstrual effluent composition (Allaire et al., 2023).

Critically, self-collection devices — menstrual cups, absorbent underwear, and tampon-based collectors — have been validated for preserving RNA and protein integrity sufficient for molecular analysis (Fasoli et al., 2021), though standardisation of collection protocols across studies remains an acknowledged challenge.

2.2 The Biochemistry of Smoking-Induced Reproductive Toxicity

Cigarette smoke contains an estimated 4,000–7,000 chemical compounds, of which several hundred are established biological toxins. Among those with demonstrated reproductive toxicity are nicotine, cotinine (the primary nicotine metabolite), cadmium, benzo[a]pyrene (BaP), and various polycyclic aromatic hydrocarbons (PAHs) (Soares et al., 2007; Augood et al., 1998). Their principal mechanism of reproductive injury is oxidative — the generation of ROS in excess of what cellular antioxidant systems can neutralise.

2.2.1 Oxidative Stress Markers

Malondialdehyde (MDA) is the most widely used biomarker of lipid peroxidation. Multiple studies have found significantly elevated MDA in the serum and reproductive fluids of smokers compared to non-smokers (Ménézo et al., 2011; El-Nemr et al., 1998). Reductions in the enzymatic antioxidants GSH, CAT, SOD, and glutathione peroxidase (GPx) have been documented in endometrial stromal cells exposed to nicotine in vitro, with corresponding MDA elevation (Yurtcu et al., 2010). These effects mirror findings in peripheral blood but are more pronounced in tissue-proximal samples, supporting the case for using a uterine-origin biofluid rather than serum.

2.2.2 Endometrial-Specific Effects

The endometrium appears especially vulnerable to smoking toxicity because of its high cellular turnover, hormonally driven remodelling, and reliance on precisely regulated gene expression for receptivity. Cigarette smoke extract (CSE) has been shown to significantly suppress *CXCL12* (stromal cell-derived factor 1) and *FGF2* (fibroblast growth factor 2), two ligands essential for endometrial receptivity and trophoblast invasion (Freour et al., 2008; Sauer & Paulson, 1992). Paradoxically, *HOXA10* — a transcription factor critical for uterine patterning — has been found upregulated in some smoking models, though this dysregulation is considered maladaptive in the context of implantation (Block et al., 2000; Miller et al., 2012).

Antioxidant response genes, including *NRF2* (Nuclear factor erythroid 2-related factor 2) and its downstream target *HMOX1* (haem oxygenase-1), are consistently activated by cigarette smoke exposure as part of a compensatory cellular defence response. Their differential expression in

smoking versus non-smoking endometrial tissue has been documented in multiple transcriptomic studies (Howe et al., 2022; Soares et al., 2007).

2.2.3 Phase I/II Metabolic Enzymes

CYP1A1 (cytochrome P450 family 1A1) is a phase I xenobiotic-metabolising enzyme activated by PAHs and dioxins, and is reliably induced in tissues exposed to cigarette smoke. Its expression in endometrial cells is considered a biomarker of local PAH exposure, as CYP1A1-mediated metabolism can generate reactive quinone intermediates that amplify oxidative damage (Buters et al., 2003). The expression of *CYP1A1* in menstrual blood cells has not, to our knowledge, been studied in the context of smoking.

2.3 Genetic Susceptibility: The NAT2 Genotype

Not all women who smoke experience equivalent reproductive toxicity, and individual variation in xenobiotic metabolism accounts for a substantial portion of this heterogeneity. The *NAT2* gene encodes N-acetyltransferase 2, a phase II enzyme that inactivates arylamine and hydrazine toxins derived from cigarette smoke. Individuals with slow-acetylator *NAT2* genotypes (common in approximately 50% of European populations) have reduced capacity to detoxify smoking-derived carcinogens, leading to greater accumulation of reactive metabolites in target tissues (Hein et al., 2000). Studies have investigated *NAT2* as an effect modifier of smoking's reproductive impact, with slow acetylators showing stronger associations between smoking and ovarian reserve reduction (Whitworth et al., 2012). This genetic dimension represents an important covariate for future mechanistic studies and is noted here as a basis for participant stratification in follow-on work.

2.4 Granulosa Cell Epigenetics and DNA Methylation

The epigenomic consequences of smoking in reproductive tissues have been most extensively studied in granulosa cells (GCs), the somatic cells directly surrounding the developing oocyte. An epigenome-wide association study identified smoking-associated differential DNA methylation in GCs at loci including *KCNQ1* and *RHBDD2*, with distinct profiles persisting in former smokers — suggesting durable epigenetic remodelling rather than a transient response (Howe et al., 2022). While GCs are not directly accessible in menstrual blood, these findings establish proof-of-concept that reproductive tract cells retain smoking-related epigenetic signatures, and make the analogous question for endometrial cells — which *are* shed into menstrual blood — a compelling and logically grounded one.

2.5 The Research Gap

The convergence of evidence reviewed above reveals a striking absence: no study has combined the accessibility of menstrual blood self-collection with the established biology of smoking-induced oxidative and transcriptional damage to answer the straightforward question of whether smoking leaves a readable chemical fingerprint in menstrual effluent. This study is designed to fill that gap.

Table 1. Comparison of sample types used in smoking–reproductive toxicity research.

Sample Type	Proximity to Endometrium	Invasiveness	Population Access	Cyclic Availability
Peripheral serum	Low (systemic)	Low (venepuncture)	Broad	Continuous
Follicular fluid	High (ovarian)	High (transvaginal aspiration)	IVF patients only	Per cycle, clinically
Endometrial biopsy	High (direct)	Moderate–High	Clinical setting	Per cycle, clinically
Menstrual blood	**High (direct shed)**	**None (self-collection)**	**Broad**	**Monthly, at home**

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3. METHODOLOGY

3.1 Study Design

This is a cross-sectional, observational, case-control study comparing menstrual blood composition between active cigarette smokers and non-smoking controls. The study will be conducted in two phases: (1) participant recruitment and menstrual blood self-collection, and (2) laboratory analysis of collected samples.

3.2 Ethical Approval

Ethical approval will be sought from the [Institutional Review Board / Ethics Committee name]. All procedures will be conducted in accordance with the Declaration of Helsinki (2013 revision). Written informed consent will be obtained from all participants prior to enrolment. Participant data will be anonymised and stored in compliance with applicable data protection legislation.

3.3 Participants

Inclusion criteria:

- Female, aged 18–40 years

- Regular menstrual cycles (21–35 days; cycle length consistent within ±3 days over the preceding 3 cycles)
- Body mass index (BMI) 18.5–30 kg/m²
- No current hormonal contraceptive use (pill, patch, hormonal IUD, implant) for ≥3 months prior to enrolment
- No current pregnancy or breastfeeding

Smoker group (n = 30):

- Self-reported active cigarette smoker for ≥1 year
- ≥5 cigarettes per day at time of enrolment
- Urinary or salivary cotinine ≥200 ng/mL (objective verification)

Non-smoker group (n = 30):

- No tobacco product use, current or historical (never-smoker)
- Cotinine <15 ng/mL
- No regular exposure to second-hand smoke (e.g., non-smoking household, non-smoking workplace)

Exclusion criteria:

- Diagnosis of endometriosis, uterine fibroids, PCOS, or other structural gynaecological pathology
- Chronic systemic illness (diabetes, autoimmune disease, renal/hepatic impairment)
- Current use of antioxidant supplements (vitamins C or E, N-acetylcysteine, CoQ10) within 30 days of collection
- Use of e-cigarettes or other nicotine delivery systems in the non-smoker group
- Inability to provide informed consent or comply with self-collection protocol

3.4 Sample Size Justification

Based on prior studies reporting MDA differences between smokers and non-smokers in reproductive tissues (effect size d ≈ 0.8–1.2), a sample of 30 per group provides >85% power at α = 0.05 (two-tailed) to detect a medium-to-large effect size. Given the novelty of the menstrual blood matrix, this initial study is designed as a proof-of-concept with the primary aim of establishing whether the signal is detectable; a larger confirmatory trial would follow.

3.5 Menstrual Blood Self-Collection Protocol

Participants will be provided with a standardised self-collection kit containing:

- A medical-grade menstrual cup (MeLuna® Sport, size M or L, allocated by self-reported flow)

- A sterile 15 mL collection tube (pre-filled with 1:1 volume RNA stabilisation solution, e.g., RNAlater™ or equivalent)
- Printed illustrated collection instructions
- A pre-labelled, insulated return mailer with a dry-ice insert (for next-day courier return)
- A brief baseline questionnaire (see Appendix A)

Collection will occur on **menstrual cycle day 1 or 2** (the first 48 hours of flow, when endometrial shedding is maximal and cell viability is highest). Participants will be instructed to:

1. Insert the menstrual cup at the onset of flow and wear it for a standardised 4-hour collection window (8:00 AM – 12:00 PM to minimise circadian confounding).
2. Carefully decant the collected effluent into the stabilisation tube, seal it, and place it immediately in the insulated mailer.
3. Arrange courier collection or deliver to the research centre on the same day.
4. Record the exact collection time, cycle day, and any deviations from protocol.

Upon laboratory receipt, samples will be split: one aliquot (500 µL) centrifuged for cell pellet (stored at −80°C for RNA extraction), one aliquot (200 µL) processed for cotinine assay, and the remaining supernatant stored at −80°C for biochemical assays.

3.6 Biochemical Assays for Oxidative Stress

All assays will be performed in triplicate on supernatant fractions of menstrual effluent. Protein concentration will be quantified first (Bradford assay) to normalise results.

Marker	**Assay Method**	**Expected Direction in Smokers**
Malondialdehyde (MDA)	TBARS (Thiobarbituric Acid Reactive Substances) colorimetric assay	↑ Elevated
Total Glutathione (GSH)	DTNB-GSSG reductase recycling assay	↓ Reduced
Catalase (CAT)	Spectrophotometric (H_2O_2 consumption rate at 240 nm)	↓ Reduced
Superoxide Dismutase (SOD)	NBT photochemical reduction inhibition assay	↓ Reduced
Total Antioxidant Capacity (TAC)	FRAP or ABTS radical cation decolorisation assay	↓ Reduced

3.7 RNA Extraction and Gene Expression Analysis

The cell pellet from centrifugation will be processed using a validated RNA extraction protocol (e.g., TRIzol® reagent, followed by RNeasy MinElute cleanup). RNA quantity and purity will be assessed by spectrophotometry (NanoDrop™). RNA integrity will be confirmed by Bioanalyzer (RIN ≥ 6 will be required for inclusion in expression analyses).

Complementary DNA (cDNA) will be synthesised using a standardised reverse transcription kit (Applied Biosystems High-Capacity cDNA Reverse Transcription Kit). Real-time quantitative PCR (RT-qPCR) will be performed using validated TaqMan probe sets for the following target genes:

Gene	**Function**	**Rationale**
CXCL12	Endometrial receptivity (chemokine signalling)	Suppressed by cigarette smoke extract in prior in vitro work
FGF2	Endometrial angiogenesis / implantation signalling	Smoking-associated downregulation documented
HOXA10	Endometrial patterning transcription factor	Paradoxical upregulation in smokers — to be interrogated
CYP1A1	PAH-induced xenobiotic metabolism	Direct biomarker of PAH exposure; not yet studied in menstrual blood
NRF2	Master antioxidant transcription factor	Compensatory activation expected in smokers
HMOX1	Haem oxygenase-1 (NRF2 downstream)	Established oxidative stress response gene

Housekeeping genes: *GAPDH* and *ACTB* (beta-actin) will serve as reference genes; stability will be confirmed using geNorm. Relative expression will be calculated by the 2^(−ΔΔCt) method.

3.8 Cotinine Verification

Cotinine — the primary metabolite of nicotine with a half-life of ~16–20 hours — will be quantified in the menstrual effluent supernatant using a validated ELISA kit (Abnova or equivalent) to (a) objectively confirm smoking status, and (b) provide a quantitative smoking intensity variable for dose-response analyses.

3.9 Participant Questionnaire

A brief self-administered questionnaire will collect:

- Smoking history (years smoked, cigarettes/day, brand type, filter status)
- Menstrual cycle characteristics (cycle length, duration of flow, dysmenorrhoea severity on a 0–10 VAS)
- Dietary antioxidant intake (validated FFQ subset: fruit/vegetable servings, supplement use)
- Physical activity (IPAQ short form)
- Alcohol and recreational drug use
- Occupation and passive smoke exposure

3.10 Statistical Analysis

All statistical analyses will be performed in R (v4.4.0) and SPSS v29. The following analyses are planned:

1. **Descriptive statistics:** Mean ± SD for continuous variables; frequency (%) for categorical. Shapiro-Wilk test for normality.

2. **Primary comparisons (smoker vs. non-smoker):**

    - Independent-samples t-test (normally distributed) or Mann-Whitney U test (non-normal) for MDA, GSH, CAT, SOD, and TAC.
    - Multiple comparison correction: Bonferroni correction applied across the five oxidative stress markers ($\alpha_{corrected} = 0.01$).

3. **Gene expression analysis:** $\Delta\Delta Ct$ values compared between groups using Mann-Whitney U. Volcano plots will be generated for visual representation.

4. **Correlation analysis:** Pearson's or Spearman's correlation between cotinine concentration and each oxidative stress marker. Cotinine will also be correlated with individual gene expression values.

5. **Covariates:** Linear regression models will include age, BMI, cycle day of collection, dietary antioxidant score, and alcohol use as covariates to isolate the independent effect of smoking status.

6. **Dose-response:** In the smoker subgroup, cigarettes-per-day and cotinine concentration will be modelled against MDA and antioxidant enzyme levels.

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4. RESULTS

Note: As this manuscript presents a proposed study protocol, no empirical results are yet available. The following section describes the data outputs and planned presentation format. The hypothesised results are presented for scientific transparency and to support grant/ethics applications.

4.1 Participant Characteristics

A summary demographics table (Table 2) will report age, BMI, mean cycle length, collection day, daily cigarettes smoked, and years of smoking for both groups. Groups will be compared for baseline equivalence (Chi-squared or t-test as appropriate).

Table 2. Anticipated participant demographics (planned format).

Variable	**Smokers (n=30)**	**Non-Smokers (n=30)**	**p-value**
Age (years), mean ± SD	—	—	—
BMI (kg/m²), mean ± SD	—	—	—
Cycle length (days), mean ± SD	—	—	—
Collection day (Day 1 or 2), n (%)	—	—	—
Cotinine (ng/mL), mean ± SD	—	—	—
Cigarettes/day, mean ± SD	—	N/A	N/A
Smoking duration (years), mean ± SD	—	N/A	N/A

4.2 Oxidative Stress Markers

Results will be presented as group means ± SD, with individual data points overlaid on box plots (Figure 1). Based on prior evidence, we anticipate:

- **MDA:** ~40–60% higher in smokers (consistent with serum-level findings; El-Nemr et al., 1998; Ménézo et al., 2011)
- **GSH:** ~25–35% lower in smokers
- **CAT:** ~20–30% lower in smokers
- **SOD:** ~15–25% lower in smokers
- **TAC:** ~30–40% lower in smokers

Whether these magnitudes translate from serum to menstrual blood is the core empirical question.

4.3 Gene Expression Results

RT-qPCR data will be presented as relative expression ($2^{-\Delta\Delta Ct}$) bar plots with 95% confidence intervals (Figure 2). Volcano plots will display $-\log_{10}$(p-value) against $\log_2$ fold change for all six target genes simultaneously.

Anticipated directional findings based on prior literature:

- ***CYP1A1*:** significantly upregulated in smokers (2–5 fold; induced by PAH exposure)
- ***NRF2*:** upregulated in smokers (compensatory antioxidant response)
- ***HMOX1*:** upregulated in smokers (NRF2 downstream target)
- ***CXCL12*:** downregulated in smokers (consistent with Freour et al., 2008)
- ***FGF2*:** downregulated in smokers (consistent with implantation signalling suppression)
- ***HOXA10*:** directionally ambiguous — both up and downregulation have been reported; the menstrual blood context may resolve this

4.4 Correlation with Cotinine

Scatter plots and Pearson/Spearman correlation coefficients will show the relationship between menstrual blood cotinine and each oxidative stress marker. A significant positive correlation between cotinine and MDA (and negative correlations with antioxidant enzymes) would constitute strong evidence that the menstrual fluid signal is dose-dependent and smoke-specific.

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5. DISCUSSION

5.1 The Novelty of the Menstrual Blood Matrix

This study's central conceptual contribution is the argument — and, prospectively, the demonstration — that menstrual blood is an appropriate and superior sample type for localised assessment of smoking-induced endometrial toxicity. The advantages are multiple. Unlike peripheral venepuncture, menstrual collection is entirely non-invasive and can be repeated

monthly. Unlike follicular fluid collection, it does not require ART procedures and is available to any reproductive-age woman regardless of fertility treatment status. Unlike endometrial biopsy, it requires no speculum, no clinic visit, and carries no risk of intrauterine infection or procedural discomfort.

The presence of shed endometrial cells — including endometrial stem cells capable of retaining tissue-specific transcriptional signatures — means that menstrual blood may, in effect, offer a monthly "biopsy by proxy" of the uterine lining. If smoking induces oxidative damage and transcriptional dysregulation at the endometrial level (as in vitro evidence strongly suggests), these signals should theoretically be detectable in the cells that the endometrium sheds each cycle.

5.2 Anticipated Findings in Context

If MDA is found to be elevated in the menstrual blood of smokers, this would extend a pattern established in serum (El-Nemr et al., 1998), follicular fluid (Ménézo et al., 2011), and in vitro endometrial cell models (Yurtcu et al., 2010) into the most clinically accessible reproductive biofluid. The magnitude of effect in menstrual blood may in fact be larger than in serum, because MDA generated by oxidative damage to endometrial cells is deposited locally in the effluent rather than diluted across the systemic circulation.

The finding of *CYP1A1* upregulation, if confirmed, would be particularly impactful: it would indicate that PAH-derived metabolites from cigarette smoke reach the uterine microenvironment at concentrations sufficient to induce xenobiotic metabolism, and that this induction is legible in shed endometrial cells. *CYP1A1* expression in menstrual blood would thereby serve both as a biomarker of smoking exposure and a mechanistic indicator of the route by which cigarette toxins damage the endometrium.

The downregulation of *CXCL12* and *FGF2* in smokers' menstrual blood would corroborate earlier cell-culture findings (Freour et al., 2008) in a more physiologically relevant context. These genes are implicated in trophoblast chemotaxis and endometrial angiogenesis, and their suppression is mechanistically linked to the higher rates of implantation failure and ectopic pregnancy in smokers (Augood et al., 1998). Detecting their suppression in menstrual blood would provide a direct, non-invasive measure of endometrial receptivity compromise.

5.3 Implications for Clinical Diagnostics

If this study's hypotheses are supported, the translational implications are substantial. A standardised menstrual blood assay panel — cotinine + MDA + *CYP1A1* expression — could potentially serve as a point-of-care tool for assessing smoking-related reproductive risk in women attending fertility clinics or general practice. Such a tool would be particularly valuable in settings where reliable self-reported smoking data is unavailable (given well-documented underreporting of smoking in fertility clinic populations; Hull et al., 2000) and where serum cotinine testing alone does not indicate the degree of tissue-level damage.

More broadly, validating menstrual blood as a reliable biomarker medium for smoking-related endometrial change would strengthen the case for its use in monitoring other environmental or pharmacological exposures — heavy metals, endocrine-disrupting chemicals, and chemotherapy toxicity among them.

5.4 Limitations

Several limitations of the proposed study must be acknowledged.

1. Cross-sectional design. This design establishes association, not causation. Longitudinal data — tracking the same women before, during, and after smoking cessation — would be needed to confirm that the observed differences are attributable to, and reversible by, changes in smoking status.

2. Sample heterogeneity. Menstrual blood composition varies with cycle day, flow intensity, phase of the cycle, and individual anatomy. Standardising collection to days 1–2 and a fixed 4-hour window mitigates this but does not eliminate biological variability. RNA yield and quality from menstrual samples can be inconsistent, and samples with RNA Integrity Number (RIN) <6 will be excluded, potentially reducing effective sample size.

3. Cell type heterogeneity. Menstrual blood contains a heterogeneous mixture of cell types — endometrial epithelial cells, stromal cells, MenSCs, immune cells, and red blood cells. Without single-cell RNA sequencing (scRNA-seq), the gene expression data reflect the population average rather than endometrial-cell-specific changes. Future work incorporating scRNA-seq would substantially improve mechanistic specificity.

4. Passive smoking and confounders. The strict exclusion of passive smokers in the non-smoking group strengthens internal validity but may limit generalisability. Dietary antioxidants, BMI, and alcohol use will be included as covariates, though residual confounding cannot be excluded.

5. Absence of a former-smoker group. The inclusion of a former-smoker cohort would allow assessment of whether oxidative stress and gene expression markers normalise after cessation — a question of high clinical relevance given the established persistence of epigenetic changes in GCs (Howe et al., 2022).

6. No gold standard comparator. Without paired serum or endometrial biopsy from the same participants, it is not possible in this design to validate menstrual blood values against established reference tissues. Future studies should incorporate matched serum sampling.

5.5 Future Directions

This study is explicitly designed as a proof-of-concept. If the primary hypotheses are confirmed, a natural progression of the research programme would include:

1. **Longitudinal smoking cessation study** — tracking the same women through cessation to determine the timeline of marker normalisation.
2. **Single-cell transcriptomics** — applying scRNA-seq to menstrual blood to resolve cell-type-specific smoking signatures.
3. **Epigenomics** — extending the GC methylation findings (Howe et al., 2022) to MenSCs from smokers.
4. **NAT2 genotyping** — sub-stratifying participants by acetylator status to explore gene-environment interactions.
5. **Point-of-care device development** — if effect sizes are sufficiently large, exploring lateral flow or electrochemical biosensor formats for clinical deployment.

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6. CONCLUSION

The scientific literature has established, with considerable confidence, that cigarette smoking inflicts meaningful biochemical and transcriptional damage on the female reproductive tract — particularly the endometrium — through mechanisms of oxidative stress, antioxidant depletion, and xenobiotic induction. What has not been tested is whether this damage is legible in the menstrual blood that the body naturally produces each month and that any woman can collect at home.

This paper proposes that it is — and that menstrual blood, properly collected and assayed, may offer the most accessible, least invasive, and most endometrially specific window yet identified into the reproductive consequences of smoking. If a woman's cigarette habit can indeed be read in her menstrual blood, the implications extend far beyond a single study: they reframe the menstrual cycle not as a monthly inconvenience but as a diagnostic opportunity — a window into uterine health that opens, month after month, without a speculum, without a needle, and without a clinic.

Future research should pursue the full scientific programme outlined here — from cross-sectional biomarker discovery, through longitudinal cessation tracking, to single-cell mechanistic studies and, ultimately, point-of-care device development. The menstrual cup, once dismissed as a mere hygiene product, may yet become a diagnostic instrument.

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APPENDIX A: PARTICIPANT BASELINE QUESTIONNAIRE

Section 1: Demographics

- Age: _____
- Highest education level: Primary / Secondary / Undergraduate / Postgraduate
- Occupation: _____

Section 2: Menstrual History

- Average cycle length (days): _____
- Average number of days of bleeding: _____
- Pain score on first day of period (0 = none, 10 = worst imaginable): _____
- Have you been diagnosed with any of the following? (tick all that apply) ☐ Endometriosis ☐ PCOS ☐ Uterine fibroids ☐ None of the above

Section 3: Smoking History *(Smoker group only)*

- How many cigarettes do you smoke per day on average? _____
- How long have you been smoking regularly? (years) _____
- Brand type (if known): _____
- Do you use a filter? ☐ Yes ☐ No
- Have you tried to quit in the past 12 months? ☐ Yes ☐ No

Section 4: Lifestyle

- On average, how many portions of fruit and vegetables do you eat per day? _____
- Do you take any vitamin or antioxidant supplements? ☐ Yes ☐ No → If yes, specify: _____
- Do you drink alcohol? ☐ Never ☐ Occasionally (<1/week) ☐ Regularly (≥1/week)
- How would you describe your physical activity level? ☐ Sedentary ☐ Lightly active ☐ Moderately active ☐ Very active

Section 5: Environmental Exposures

- Does anyone in your household smoke? ☐ Yes ☐ No
- Are you regularly exposed to cigarette smoke at work? ☐ Yes ☐ No
- Do you use any of the following? (tick all that apply) ☐ E-cigarettes/vapes ☐ Hookah/shisha ☐ Smokeless tobacco ☐ None

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APPENDIX B: SUMMARY OF PLANNED ASSAY KITS AND EQUIPMENT

Analysis	**Kit / Instrument**	**Supplier**
MDA (TBARS)	TBARS Assay Kit	Cayman Chemical / Sigma-Aldrich
Total GSH	Glutathione Assay Kit (DTNB recycling)	Sigma-Aldrich
Catalase	Catalase Activity Assay Kit	Abcam (ab83464)
SOD	SOD Assay Kit (WST-1)	Sigma-Aldrich
TAC (FRAP)	Total Antioxidant Capacity Assay Kit	Abcam (ab65329)
Cotinine (ELISA)	Nicotine Metabolite ELISA Kit	Abnova
RNA extraction	TRIzol® + RNeasy MinElute	Thermo Fisher / Qiagen
RNA quantification	NanoDrop™ One	Thermo Fisher
RNA integrity	2100 Bioanalyzer	Agilent
cDNA synthesis	High-Capacity cDNA RT Kit	Applied Biosystems

Analysis	Kit / Instrument	Supplier
RT-qPCR	TaqMan probes; QuantStudio 6 Flex	Applied Biosystems
Protein quantification	Bradford Protein Assay	Bio-Rad

End of Manuscript

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